Effect of Fluid Resuscitation on Acute Skeletal Muscle Ischemia-Reperfusion Injury after Hemorrhagic Shock in Rats

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BACKGROUND: Severe extremity wounds with vascular injury are common in military trauma, and tourniquets are commonly used for hemorrhage control. The complications of tourniquet use in the setting of trauma are not well studied. This study investigated the combined effect of hemorrhagic shock and fluid resuscitation with Hextend (HX; BioTime, Inc) or lactated Ringer's (LR) on skeletal muscle subjected to tourniquet-induced ischemia-reperfusion injury.

STUDY DESIGN: Thirty male Sprague-Dawley rats underwent 33% arterial hemorrhage followed by 3 hours of tourniquet application. Before reperfusion, 10 animals each were resuscitated with lactated Ringer's (3 times shed volume) or HX (shed volume). Ten control animals received no resuscitation. Rats were euthanized 2 hours after tourniquet release and the tibialis anterior and medial gastrocnemius muscles were examined for edema (muscle wet weight) and viability (nitroblue tetrazolium reduction). Contralateral muscles served as controls for each animal, with results expressed as the ratio of the tourniquet limb to contralateral limb values.

RESULTS: The tibialis anterior and medial gastrocnemius muscles in all groups experienced edema, with all weight ratios greater than one. Resuscitation with HX resulted in significantly ($p < 0.05$) greater edema than did no resuscitation in both muscles and greater edema than with lactated Ringer's in the medial gastrocnemius. All groups experienced a loss of viability as well, with nitroblue tetrazolium reduction ratios less than one. Resuscitation with HX resulted in significantly less viability loss than did no resuscitation in the medial gastrocnemius. No significant differences in viability were seen in the tibialis anterior.

CONCLUSIONS: Resuscitation with HX or lactated Ringer's does not adversely affect muscle viability in ischemia-reperfusion injury. HX may be a better clinical choice when skeletal muscle ischemia-reperfusion injury is a risk, despite greater edema. (J Am Coll Surg 2006;202:888–896. © 2006 by the American College of Surgeons)

Extremity trauma is the most frequent source of injury on the modern battlefield.[1-4] Military extremity wounds tend to result from very high energy mechanisms and are typically more severe than the extremity injuries seen in civilian trauma. Major extremity vascular injuries are common, and exsanguination from major extremity vascular injury is considered a leading cause of preventable battlefield mortality.[1,5,6] Though uncommon, exsanguination from isolated extremity injuries does occur in civilian trauma.[7] To address the prevention of mortality from bleeding extremities, United States military authorities have promoted the liberal use of tourniquets for control of severe extremity hemorrhage on the battlefield.[8-10]

Tourniquets, when properly designed and used, are very effective at eliminating distal blood flow.[10,11] As a result, they are not benign interventions, and potentially significant complications can be associated with their use. After an extended application time, the ischemia associated with the proper use of a tourniquet can result in ischemia-reperfusion injury (I-R) to skeletal muscle once the tourniquet is released. It has been demonstrated

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Abbreviations and Acronyms

HX = Hextend
I-R = ischemia-reperfusion injury
LR = lactated Ringer's
MAP = mean arterial pressure
MG = medial gastrocnemius
NR = no resuscitation
TA = tibialis anterior

that limb muscles can exhibit significant edema and loss of cellular viability after a tourniquet-induced ischemic period of 2 hours or more.[12-14]

Tourniquet use in the presence of severe extremity trauma typically occurs in the setting of significant blood loss from the injured extremity and possibly other sites. Clinically, patients manifest hemorrhagic shock and require fluid resuscitation before removal of the tourniquet and attempted revascularization and salvage of the affected extremity. The combined effect on skeletal muscle of tourniquet-induced I-R and hemorrhagic shock with fluid resuscitation has not been investigated experimentally. Although skeletal muscle appears to escape significant damage in the setting of hemorrhage alone,[15] it is possible that the combination of hemorrhagic shock and fluid resuscitation may alter the pathophysiology of I-R. Previous findings using our institution's model of skeletal muscle I-R have revealed that a 33% arterial hemorrhage before tourniquet application results in an acute decrease in muscle edema but no significant effect on the loss of muscle viability seen with the I-R insult alone after 2 hours of reperfusion.

In other models of skeletal muscle I-R, the hydroxyethyl starch compounds found in some resuscitation fluids have been shown to alter the muscle injury profile.[16-18] Fluids containing these molecules may have potential as therapy for skeletal muscle I-R in addition to their primary role as circulatory volume expanders. Because initial care of trauma patients with evidence of hemorrhage includes intravenous administration of fluid, we sought to investigate the impact of fluid selection on the pathophysiology of tourniquet-induced I-R. This study evaluated the combined effect of hemorrhagic shock and fluid resuscitation with lactated Ringer's (LR; Baxter Medical) or Hextend (HX, high molecular weight hydroxyethyl starch 6% in buffered electrolyte dextrose solution; BioTime, Inc) on skeletal muscle subjected to tourniquet-induced I-R.

METHODS

Animal care

All animal protocols were approved by the United States Army Institute of Surgical Research Animal Care and Use Committee. This study adhered to National Institutes of Health guidelines for the care and use of laboratory animals (DHHS Publication, NIH, 86 to 23). Adult male Sprague-Dawley rats weighing 402 ± 28 g were obtained from a single vendor (Harlan). Animals were housed in an Association for the Assessment and Accreditation of Laboratory Animal Care–approved facility and were provided with food and water ad libitum before all procedures. All procedures were performed under 1.5% to 2.5% isoflurane anesthesia, adjusted to maintain a surgical plane. Pain was controlled with buprenorphine, 0.1 mg/kg, administered through intraperitoneal injection.

Surgical procedures and monitoring

Rats were anesthetized, had both hind limbs shaved, and were instrumented with a rectal temperature probe (Physiotemp Instrument, Inc). The carotid artery was cannulated with a PE50 polyethylene catheter internally coated with anticoagulant (TDMAC-Heparin 2%; Polysciences, Inc). Animals were placed supine on a warm water flow temperature-regulated bed (EX-212; Euthanex Corp) and core temperature was maintained at 37° ± 1°C. The catheter was used for blood sampling, hemorrhage, and continuous monitoring of mean arterial pressure (MAP) using a Labview-based computerized data acquisition system (National Instruments).

Animals were hemorrhaged at a rate of 1 mL/min to a volume equal to 33% of their blood volume. To determine the degree of hemorrhagic shock and anemia, measurements of arterial pH and hemoglobin concentration were taken at baseline and 2 hours after hemorrhage (I-STAT 1; Abbott Laboratories). A pneumatic digital tourniquet (model DC1.6; DE Hokanson, Inc) was placed as proximal as possible around a randomly selected hind limb. This leg was elevated above the level of the heart for 5 minutes before tourniquet inflation. The cuff was then inflated and pressure maintained at 250 mmHg using a cuff inflator (model E20; DE Hokanson, Inc) and air source (model AG101; DE Hokanson, Inc).

Resuscitation protocol and reperfusion

A tail vein was catheterized using a 22-gauge angiocatheter (Medex). Resuscitation was timed such that it was complete just before tourniquet release. A volume of LR (n = 10) equal to three times the shed blood volume was administered through the tail vein at 1 mL/min. A volume of HX (n = 10) equal to that of the shed volume was administered at 0.33 mL/min to equal the infusion period of the LR-treated rats. Control animals (NR, n = 10) underwent hemorrhage, tourniquet placement, and tail vein cannulation, but did not receive fluid resuscitation.

Tourniquets were left in place for 180 minutes. After release, animals were allowed to recover from anesthesia and were returned to their cages for a 120-minute reperfusion period. After the 2-hour reperfusion period, rats were euthanized with an intracardiac injection of pentobarbital, and bilateral hind limb dissection for end-point determination was performed.

End-point determination

The medial gastrocnemius (MG) and tibialis anterior (TA) muscles of the tourniquet limbs and contralateral limbs were individually dissected and removed with their tendons. Whole muscle weights were obtained on a microbalance (MT-5; Mettler Toledo, Inc). Muscle viability was determined using the spectrophotometric nitroblue tetrazolium reduction technique according to the method of Powell and colleagues.[19]

Statistical analysis

To control for differences in animal weights, experimental results were standardized within each animal to the contralateral limb. The ratios of tourniquet limb to contralateral limb muscle weight and viability by nitroblue tetrazolium reduction were calculated for each muscle by dividing tourniquet limb values by the corresponding contralateral muscle values. Statistical analyses were performed using commercially available software (SigmaStat 3.1; Systat Software, Inc). Group mean ratios for experimental groups were compared using analysis of variance with appropriate posthoc tests. Differences were considered to be significant at $p < 0.05$. All data are presented as mean ± standard error of the mean.

RESULTS

All 30 rats survived for the entire 5.5-hour experimental period. The mean hemorrhaged volume was 8.73 ± 0.12 mL and resulted in a significant drop in MAP in all three groups: from a baseline of 99 ± 2 mmHg to a nadir of 38 ± 3 mmHg ($p < 0.05$). Blood pressure began to rise immediately after hemorrhage, and all groups achieved MAP near their baseline by the time fluid resuscitation was initiated. During fluid infusion in both the LR and HX groups, MAPs remained stable near their baseline values. A drop in MAP on tourniquet release of 22 ± 8 mmHg was seen in the NR group. This decrease was significantly ($p < 0.05$) greater than both the 4 ± 3 mmHg seen in the LR animals and the 6 ± 2 mmHg seen in the HX animals. The HX group's MAP was significantly higher than that of the NR animals during early reperfusion ($p < 0.05$). Blood pressure profiles during the experiment were consistent with a state of compensated shock. All groups experienced compensatory vasoconstriction, reaching and maintaining near-baseline MAPs after hemorrhage. The LR and HX resuscitated groups experienced accommodating vasodilation during resuscitation, allowing for maintenance of MAP despite the infusion of fluid. No such vasodilation was apparent in the NR group, and there was no significant change in MAP in any group during the resuscitation period (Fig. 1).

Two hours after hemorrhage, all groups manifested physiology consistent with hemorrhagic shock, exhibiting a significant drop in arterial pH from 7.35 ± 0.01 to 7.28 ± 0.02 ($p < 0.05$). Significant anemia was also present in all groups, with hemoglobin concentration decreasing from a baseline of 13.5 ± 0.11 g/dL to 8.9 ± 0.12 g/dL ($p < 0.05$) 2 hours after hemorrhage (Table 1).

There were no significant differences between groups in contralateral limb muscle weights, indicating minimal effect of resuscitation with LR or HX on muscles not subject to I-R. All groups' tourniquet limb muscles experienced edema, as measured by tourniquet limb to contralateral limb weight ratios greater than one. Resuscitation with HX resulted in significantly greater TA edema than did NR ($p < 0.05$) and significantly greater MG edema than did both LR and NR ($p < 0.05$, Fig. 2). In addition to edema, all tourniquet limb muscles experienced an

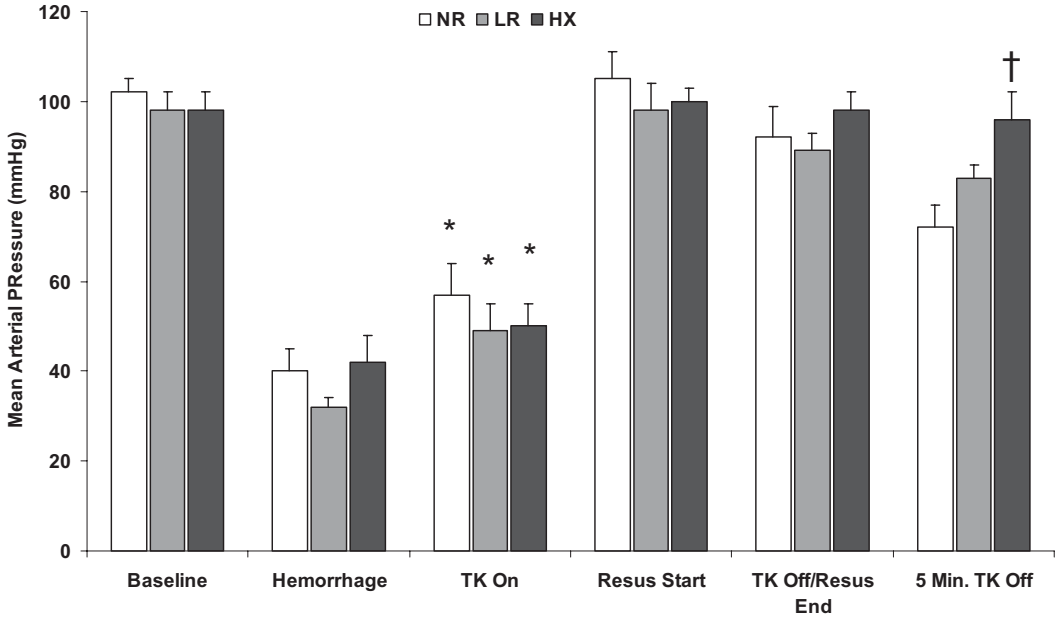

Figure 1. Mean arterial pressure (MAP) at experimental events. All groups experienced a large drop in MAP with hemorrhage and were significantly (*, $p < 0.05$) hypotensive relative to their baseline at the time of tourniquet (TK) application. MAPs had normalized spontaneously before the start of resuscitation (Resus). No group experienced a significant change in MAP during the resuscitation period. Hextend-resuscitated animals had a significantly (†, $p < 0.05$) higher MAP after 5 minutes of limb reperfusion than did nonresuscitated animals. HX, Hextend; LR, lactated Ringer's; NR, no resuscitation.

approximately 60% loss of viability, manifested by tourniquet limb muscle to contralateral limb muscle nitroblue tetrazolium reduction ratios less than 1. The MG lost less than 50% viability with HX resuscitation, a significantly smaller loss than that in the NR group ($p < 0.05$, Fig. 3).

DISCUSSION

The dual goals of fluid resuscitation after hemorrhage are to replace lost blood volume and restore tissue perfusion. Despite years of animal research and treatment of countless patients, the optimal resuscitation fluid and strategy remain unknown. Debate about the use of crystalloid or colloid has continued for more than 50 years. Large volume crystalloid resuscitation with LR is the current standard of trauma care and can be expected to be effective in most situations.[20] Studies in animal shock models have generally used two to four times the shed blood volume of LR to resuscitate from hemorrhage, based on early work by Dillon and associates in dogs.[21] Additional rationale for this fluid volume is based on observations that only about 25% of the infused LR remains in the circulation after 1 hour.[22,23]

It is generally accepted that colloid solutions are more efficient than crystalloids as resuscitation fluids because of their ability to retain intravascular water.[24] Historically, a 1:1 replacement volume of hetastarch to shed blood has been used in animal models. This experiment was designed to test the combined effect of hemorrhagic shock and fluid resuscitation with LR or HX on skeletal muscle subjected to tourniquet-

Table 1. Physiologic Parameters Before and 2 Hours after Hemorrhage

	pH		Hemoglobin (g/dL)	
Group	**Baseline**	**Hemorrhage**	**Baseline**	**Hemorrhage**
No resuscitation	7.35 ± 0.02	7.26 ± 0.02	13.4 ± 0.2	9.1 ± 0.2
Lactated Ringer's	7.35 ± 0.03	7.29 ± 0.04	13.6 ± 0.2	8.8 ± 0.3
Hextend	7.36 ± 0.02	7.28 ± 0.03	13.4 ± 0.2	9.1 ± 0.2

All groups manifested physiology consistent with hemorrhagic shock with a drop in arterial pH and anemia with a drop in hemoglobin concentration.

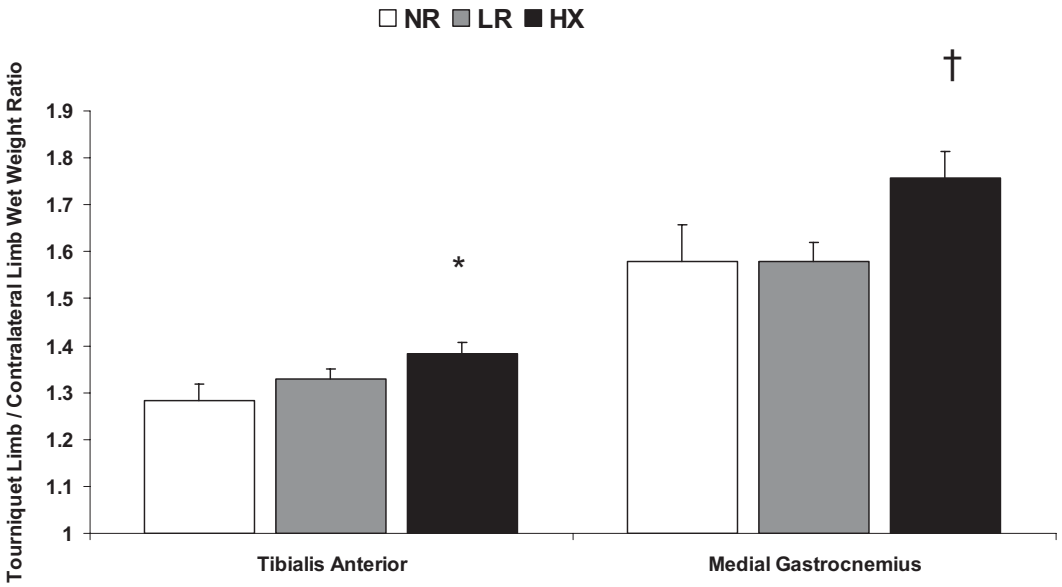

Figure 2. Edema in study muscles. Data (mean ± SEM) are presented as the ratio of the weight of the muscles from the tourniquet limb to those from the contralateral limb. Both muscles in all groups experienced edema, with ratios greater than one. Hextend (HX) resuscitation resulted in significantly greater edema than no resuscitation did in the tibialis anterior (*, $p < 0.05$) and significantly greater edema than did lactated Ringer's (LR) and no resuscitation (NR) in the medial gastrocnemius (†, $p < 0.05$).

induced I-R. We measured the effect by quantifying muscle edema and loss of cellular viability in relation to the muscles of the contralateral, unaffected limb. The rat was chosen for this model because of the extensive body of comparative literature available in the field of skeletal muscle I-R in small rodents. Although the field of experimental resuscitation has moved to more complex, larger animal models, we believed that the interaction of hemorrhage, fluid replacement, and tourniquet-induced skeletal muscle I-R could be best studied in this animal.

We chose to examine two clinically relevant muscles that are histologically and biochemically similar, but are located in different anatomic compartments.[25] The TA has functional importance for dorsiflexion of the foot and ambulation in the rat, as it does in the human. This muscle is located in the anterior compartment of the rat hind limb, which, as in the human, is relatively noncompliant.[26] The MG is important for pedal plantarflexion and is located in the superficial posterior compartment, which is larger than the anterior and more compliant.[26] Various authors have demonstrated that the predominant fiber type composition of skeletal muscles influences their response to an I-R insult.[27-29] Given that both the TA and the MG are predominantly composed of type II (fast twitch) muscle fibers, we expected that any difference in pathophysiologic response to I-R between the TA and MG would be based on the difference in anatomic compartment.

The tissue edema seen during acute reperfusion of skeletal muscle after ischemia is caused by increased microvascular permeability that results from ischemic muscle-generated free radical endothelial damage, primarily in postcapillary venules.[30-32] These effects begin immediately on reperfusion as a result of an initial hyperemic response to restoration of circulation and are termed the "reflow" phenomenon. Intravenous infusion of 6% hydroxyethyl starch compounds similar to HX before skeletal muscle reperfusion has been shown to attenuate the dysfunction of microvascular permeability during early reperfusion.[17-18] Our results demonstrated that isovolemic resuscitation with HX before reperfusion increased muscle edema more than with no resuscitation in both the TA and the MG. This finding is likely the result of leakage of some of the circulating hydroxyethyl starch molecules into the interstitial space, drawing plasma and creating edema through a colloid osmotic effect. Unlike the previous studies, we administered HX during a state of compensated hemorrhagic shock, which may have had an additive adverse

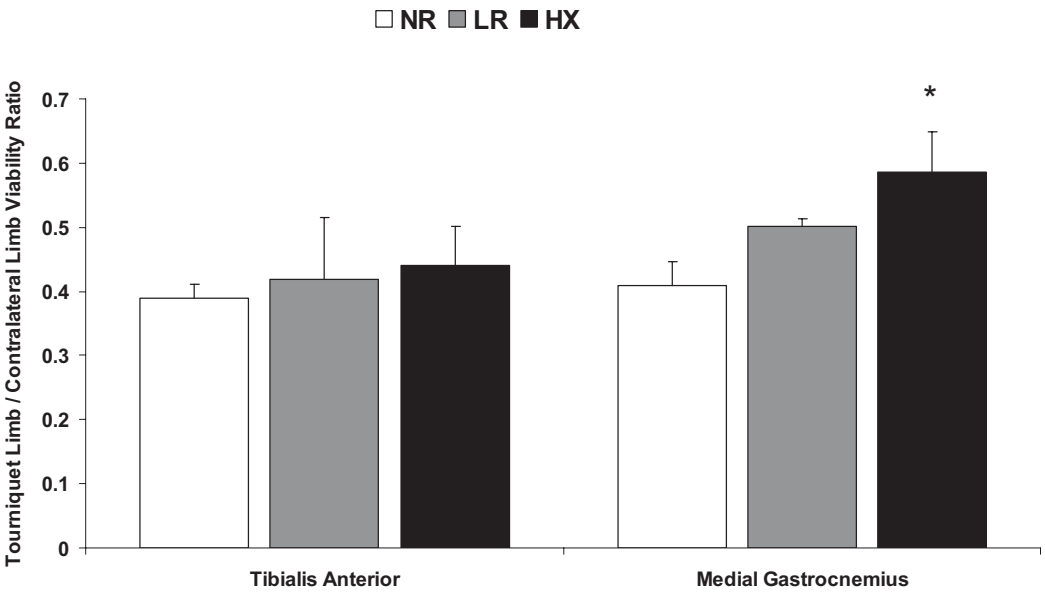

Figure 3. Viability loss in study muscles. Data (mean ± SEM) are presented as the ratio of the viability of muscles from the tourniquet limb to those from the contralateral limb. Both muscles in all groups experienced loss of viability, with ratios less than one. Hextend (HX) resuscitation resulted in significantly greater remaining viability than no resuscitation did in the medial gastrocnemius (*, $p < 0.05$). LR, lactated Ringer's; NR, no resuscitation.

effect on endothelial barrier function. Additionally, the transition from the vasoconstrictive state of shock compensation after hemorrhage to vasodilation during resuscitation may have worsened the hyperemia during initial reperfusion.

Resuscitation with LR at three times the shed volume did not lead to a further increase in edema in either muscle compared with the no fluid group. Infusion of HX has been demonstrated to increase plasma volume to a greater extent than infusion of isotonic crystalloid solutions.[33,34] In addition, the extracellular water increase seen after an infusion of HX exceeds that of the infused volume, and the effects on plasma volume are persistent for hours after infusion.[33] It is likely that the increase in tissue edema seen in the muscles of HX-resuscitated animals over those resuscitated with LR in part represented the effect of increased circulating volume delivered at a higher blood pressure to damaged muscle microvasculature during the initial, hyperemic stage of reperfusion.

In both the TA and the MG, viability loss with either LR or HX resuscitation was no greater than the viability loss in nonresuscitated animals. This suggests that there was no detrimental effect of either resuscitation protocol on muscle viability after hemorrhagic shock. In the MG, despite an increase in muscle edema, resuscitation with HX allowed for conservation of tissue viability over no resuscitation. This protective effect of HX resuscitation was likely related to an improvement in nutritive perfusion of the affected muscle, as has been seen in models of skeletal muscle trauma and hemorrhagic shock.[35] After the initial hyperemic "reflow" phenomenon, reperfusing muscle tissue becomes vulnerable to microvascular occlusion and continued hypoperfusion. This occlusion, termed the "no-reflow" phenomenon, is believed to be multifactorial, including extravascular compression by surrounding edematous tissues, endothelial cell edema, and leukocyte plugging.[12,36] It has been demonstrated that microvascular occlusion pressure is inversely related to diastolic blood pressure.[37] In this study, HX resuscitation may have facilitated delivery of a greater intravascular volume at a greater pressure throughout reperfusion, allowing continued perfusion of muscle tissue despite extravascular and endothelial edema. Additionally, neutrophil binding to thrombin-stimulated endothelial cells has been shown to decrease in the presence of hydroxyethyl starches such as HX.[17] Such a decrease in this experiment might have resulted in reduced leukocyte plugging and increased perfusion during the "no-reflow" period.

As mentioned previously, in both the TA and MG, resuscitation with HX resulted in greater edema than with LR, but this difference was significant only in the MG. The discrepancy in edema between the two muscles may relate to the relative compliance of their anatomic compartments, with edema in the TA being limited by increased compartment pressure; the more compliant posterior compartment allowed continued extravasation of fluid in the MG. The TA did not demonstrate conservation of viability with resuscitation, suggesting that it was unable to accept increased perfusion because of the constraints of the noncompliant anterior compartment. The increase in muscle edema seen in the HX-resuscitated animals may be of clinical concern at longer reperfusion time points than the 2 hours of this experiment. We did not see a concomitant decrease in viability with increase in edema, but significant muscular edema may lead to elevated compartment pressures and potentially development of muscle-threatening compartment syndrome. We are currently performing experiments with reperfusion times out to 2 days to investigate this phenomenon. The clinical significance of this experiment lies in the fact that significant muscle edema was seen in all groups regardless of resuscitation, suggesting that in patients at risk for tourniquet-induced I-R, liberal application of fasciotomy should be considered.

The hemorrhagic shock-tourniquet-resuscitation-reperfusion protocol used in this experiment represents a clinical scenario encountered in modern battlefield trauma. In such a situation, a casualty suffers severe extremity injury, causing significant hemorrhage and shock. A tourniquet is applied to the affected limb, occluding all distal blood flow and resulting in the rapid cessation of hemorrhage. The resulting ischemic time is variable and relates to the tactical situation and the timing of evacuation to surgical care at a field hospital. Both LR and HX are currently carried by field medical personnel and stocked at in-theater hospitals. In a stable casualty, intravenous fluid resuscitation is frequently delayed until a field hospital is reached and preparations are made for surgical care of the extremity after tourniquet removal. Despite severe injuries, many extremities subject to battlefield tourniquet use are salvaged,[38] and attempted salvage is currently the standard of care at United States field hospitals.

The results of this study should be considered within the context of its limitations, the principal one being the acute nature of the data. The effects that we observed at 2 hours of reperfusion might not be representative of those present during longer reperfusion times. In addition, we looked only at tissue end points and did not examine the functional status of the muscles subjected to the combined hemorrhagic shock, I-R, and resuscitation. Without these data, it is unclear whether the amounts of edema and viability loss seen were clinically significant. Studies of muscle function are the subject of ongoing work in our laboratory.

The relative infrequency in civilian trauma of injuries that might benefit from tourniquets has resulted in great controversy, an aversion to their clinical use, and a subsequent lack of basic science research dedicated to issues arising from the use of tourniquets in trauma.[10,39] Nearly all experimental literature on the subject of tourniquet-induced skeletal muscle I-R models the use of tourniquets for elective orthopaedic operations. With the frequency in modern warfare of severe extremity trauma and widespread use of tourniquets for hemorrhage control, experimental data about the effect of tourniquet use in trauma are valuable. Historically, changes in battlefield medicine have often translated into changes in the treatment of civilian trauma as military providers enter civilian practice.[40] If tourniquet use becomes more common in the acute treatment of civilian trauma patients, the value of this experimental data would translate as well.

In summary, we concluded that 3 hours of tourniquet use in hemorrhaged rats resulted in muscle edema and reduced viability in affected muscles at 2 hours of reperfusion. Fluid resuscitation did not adversely affect muscle viability after tourniquet application. HX resuscitation resulted in increased viability over no resuscitation in the MG, and given that a smaller volume of this colloid is required for volume expansion than isotonic crystalloid such as LR, it may be a better logistical choice for intravascular replacement when skeletal muscle I-R is a risk, despite an increase in edema. Additional study into the subacute muscle viability and longterm functional status of limbs subjected to the combined effect of hemorrhage, resuscitation, and tourniquet-induced I-R is warranted.

Author Contributions

Study conception and design: Kauvar, Dubick, Walters
Acquisition of data: Kauvar, Walters
Analysis and interpretation of data: Kauvar, Baer, Dubick, Walters
Drafting of manuscript: Kauvar
Critical revision: Dubick, Baer, Walters

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